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Inventor Kozikowski et al.
For CYCLIC DIPEPTIDE COMPOUNDS (BICYCLIC 2,5-DIKETOPIPERAZINE COMPOUNDS) AND THEIR USES

(X) Affidavit/Declaration of co-inventor Alan I. Faden
( ) Amendment
( ) Application____Pages
( ) ____ Claims ____ Drawings
( ) Appeal, Notice of
( ) Assignment
( ) Brief (in triplicate)
( ) Declaration & Power of Attorney
( ) Design Application
( ) Disclaimer
( ) Disclosure Statement
( ) w/refs. ( ) w/o refs.
( ) Drawings Formal
____Sheets ____Figures

( ) Fee Address Indication Form
( ) Fee Calculation
( ) Issue Fee Transmittal
( ) Letter
( ) Oral Hearing Req./Confirm.
( ) Petition to Extend Time
( ) Pet. under 37 C.F.R.
( ) Power of Attorney
____Associate____w/Revocation
( ) Sequence Listing w/Computer Readable and Paper Copies
( ) Small Entity Statement
( ) Status Letter
( ) Transmittal Letter

Other: Exhibits 1-7; and return postcard

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EXHIBIT B

Express Mail No.: EL 451 593 048 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Kozikowski et al.

Application No.: 09/022,184 Group Art Unit: 1654

Filed: February 11, 1998 Examiner: Anish Gupta

For: CYCLIC DIPEPTIDE COMPOUNDS (BICYCLIC 2,5-DIKETOPIPERAZINE COMPOUNDS) AND THEIR USES

Attorney Docket No.: 9328-0005-999

TRANSMITTAL SHEET

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicants submit herewith an executed Declaration under 37 C.F.R. § 1.132 by co-inventor Alan I. Faden, with Exhibits 1-7.

Applicants believe that no fees are due in connection with the submission of this Declaration. However, the Commissioner is authorized to charge any required fee in connection with this submission or credit any overpayment to Pennie & Edmonds LLP Deposit Account No. 16-1150. A copy of this sheet is enclosed.

Respectfully submitted,

Date November 2, 1999

Samuel B. Abrams by Beth Levin Reg No 42,813 42,813

Samuel B. Abrams, Esq. (Reg. No.)

PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, New York 10036-2711
(212) 790-9090

Enclosures

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Kozikowski *et al.*

Serial No.: 09/022,184 Group Art Unit: 1654

Filed: February 11, 1998 Examiner: Anish Gupta

For: CYCLIC DIPEPTIDE COMPOUNDS (BICYCLIC 2,5-DIKETOPIPERAZINE COMPOUNDS) AND THEIR USES

Attorney Docket No.: 9328-0005-999

DECLARATION OF ALAN I. FADEN UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, ALAN I. FADEN, declare that:

1. I am a co-inventor of the subject matter disclosed and claimed in the above-identified patent application.

2. I currently hold the position of Professor of Neuroscience, Neurology and Pharmacology at Georgetown University Medical Center, Georgetown University, Washington D.C., the assignee of the above-identified application, where I have been employed since 1991. I received an M.D. from the University of Chicago in 1971, and I have been working in the field of neurology since 1972. To date, I have published my research in more than 275 papers and chapters. My technical experience and selected publications are summarized in my *Curriculum Vitae*, which is appended hereto as Exhibit 1.

3. I have reviewed the above-identified patent application and the outstanding Office Action dated April 2, 1999. I understand that the Examiner has rejected Claims 11-17, 20, 22-27, and 32 under 35 U.S.C. §112, first paragraph, on the grounds that the specification does not provide an enabling disclosure for the claimed disorders and injuries, and for the enhancement of cognitive function.

I understand that the Examiner has made the 35 U.S.C. §112, first paragraph, rejection on the basis that the search for cognitive-enhancing therapy has proved elusive. Further, I understand that the Examiner believes that the claims are inclusive of regeneration of neurons and that CNS neurons are incapable of mounting a regenerative response. Also, I understand that the Examiner does not believe that the specification provides sufficient guidance to allow for the treatment of all claimed neurological disorders and CNS injuries. Finally, I understand that the Examiner alleges that an art recognized model that would clearly demonstrate that the compounds would be effective in the treatment of Alzheimer's does not exist.

4. Based upon the experiments conducted under my supervision and disclosed in the specification, additional data provided in this affidavit, and my 27 years of experience as a researcher in the field of neurology, I believe that methods of treatment, as recited in Appendix A of the response to the April 2, 1999 Office Action, are fully enabled by the specification. I address the reasons that the Examiner gave in the lack of enablement rejection individually in the following sections.

5. I believe the data provided in the specification effectively addresses the Examiner's concern that cognitive-enhancing therapies have proven to elusive. The specification discloses that exemplary compounds of the invention markedly enhance cognitive function in rats with chronic brain injury. The assays used in the specification to demonstrate such enhanced cognitive function are well accepted in the art. *See, e.g.*, Morris, 1984, J. Neurosci. Meth. 22:47-60; Hamm *et al.*, 1996, J. Neurotrama 13:317-323. Furthermore, prior traumatic brain injury is one of the well-recognized epidemiological factors of Alzheimer's disease. Therefore, these assays, alone, demonstrate a high likelihood that the disclosed compounds may be useful as a cognitive-enhancing therapy for indications such as Alzheimer's disease.

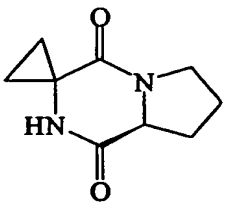
6. Contrary to the Examiner's position, I believe that the claimed methods are not necessarily inclusive of regeneration of neurons. It is now well-established that mammalian neurons can and do regenerate, and functional recovery has been clearly demonstrated in a variety of models, including experimental spinal cord injury. There is no absolute requirement for neuronal regrowth in order to effect treatment of the claimed neurological disorders and CNS injuries. Rather, it is well accepted in the art that preventative treatments are efficacious in alleviating the claimed neurological disorders and CNS injuries. It is well appreciated in the art that proper control of the complex biochemical cascade that is typical of neurological disorders and CNS injury provides a very promising route for minimizing their detrimental effects. For example, inhibition of the acute biochemical cascade, initiated by trauma, that leads to subacute cell death is a concept that is very well-established in the literature and I have written substantially on the subject, both in terms of primary papers and reviews. In addition, this principle of secondary biochemical

injury has been clearly established in human spinal cord injury and multiple therapies, including two from our own laboratory, have been shown to improve recovery after human spinal cord injury. We have clearly established in multiple, clinically relevant models of human brain injury and in different species that acute administration of our compounds substantially increase recovery. Moreover, they increase not only motor recovery, as suggested by the Examiner, but also spatial learning and memory, indicating preservation of neurons in the hippocampus. For a review of secondary injury responses please see Faden, 1996, Pharmacology & Toxicology 78:12-17.

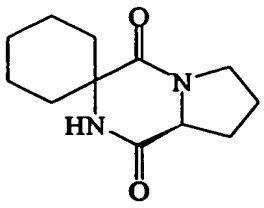
7. I believe that the specification provides sufficient guidance to allow for the treatment of all claimed neurological disorders and CNS injuries. We do not claim treatment of all CNS injuries. The claims in the accompanying response have been modified to reflect the fact that we only claim head injuries, spinal cord injury, and stroke. It is now well-established that the same biochemical and metabolic mechanisms leading to neuronal cell death after stroke, head injury, and spinal cord injury are largely identical; indeed, it is common in the pharmaceutical industry to generalize therapeutic strategies across those three classes of disorders. Further, we provide *in vitro* evidence in this affidavit showing that our compounds block neuronal cell death after insults specifically implicated in spinal cord injury and stroke, namely excitotoxic (glutamate-induced) and free radical-induced injuries. In addition, we show protective effects using an *in vitro* model of ischemia (stroke)-oxygen glucose deprivation. I believe that the data provided in the specification and this affidavit indicate that our compounds block subacute death of neurons resulting from the primary response and secondary reactive response of the tissue rather than by producing a regenerative response. For a review of current views in the field please see Faden, 1996, *Id.*

8. I believe that the Examiner's argument that no art accepted animal model exists for Alzheimer's disease is not a fair assessment of the art. No animal model predicts human disease in all regards. Furthermore, a number of animal models of Alzheimer's disease have been developed over the last several years that reflect, to a significant degree, critical pathologic and behavioral characteristics of the disorder. These models are being used to examine therapeutic strategies.

9. In addition to the experimental data provided in the specification, I have supervised additional experiments using compounds disclosed in the specification. These additional experiments further establish that the compounds of the present invention are enabled for the claimed disorders and injuries, and for the enhancement of cognitive function. The compounds used in the assays described in this affidavit have the same designations that were provided in the specification. Namely:



Compound 1



Compound 2

10. The experiments described in this affidavit were conducted using neuronal-glial co-cultures prepared as detailed below. Assessment of neuronal cell death was assayed by measuring lactase dehydrogenase (LDH) as detailed below.

Neuronal-glial Co-cultures. Glial cultures were prepared from neonatal rat cortex dissociated in supplemented Hank's Salts with magnesium and calcium deleted. Cells were

seeded (0.25 hemisphere/plate) in supplemented MEM (sMEM) with Earle's salts on 96-well plates. After 9 days *in vitro* (DIV), neocortex from day 18 gestation rat embryos was dissociated and seeded (2-2.5x10^6 cells/plate) on the glial cultures in solutions. Four DIV after the second seeding, cytosine arabinoside was added to the feeding medium, to stop proliferation of non-neuronal cells. Thereafter, cells were fed twice weekly until used for experiments on 18-21 DIV.

Assessment of Injury and Compound Effects. Cell death was assessed 16-18 hours after injury induction via assay of lactate dehydrogenase (LDH) released into the culture medium. In each of the assays described in this affidavit, fifty percent of the media was removed and diluted 1:3 with LDH assay reagent (5 mM β-NAD, 25 mM lactic acid, 0.3% bovine serum albumin, 100 mM TRIS, 0.9% NaCL, pH 8.45). Optical density at 340 nM was measured over 250 seconds at five second intervals for a total of fifty readings. The slope of the absorbance curves generated over 250 seconds represented LDH activity. Results reflect the difference between LDH release induced by injury and LDH released by uninjured control cultures. Data were expressed as percent of LDH release in treated cultures compared to that induced by injury alone. In all exhibits, bars represent the means ± standard error measurements (SEM) for *n*-25-30 wells per condition. Effect of treatment with compound vs. vehicle was assessed using unpaired t-tests.

11. Substantial evidence from many laboratories indicates that central neurotoxicity mediated by the neurotransmitter glutamate can contribute to several forms of central nervous system damage. *See, e.g.*, Buisson and Choi, 1995, Neuropharmacology 34(8):1081-1087. Accordingly, I supervised an experiment ("Excitotoxic Injury") to test the

ability of an exemplary compound of the present invention to increase survival of neuronal-glial co-cultures incubated with glutamate.

Excitotoxic Injury Cells were prepared as described in point 10 washed with sMEM, and incubated in 1 μM compound 2 ("2") or vehicle ("Control") for thirty minutes. Cultures were washed again and then incubated with or without compound 2 in the presence of 500 μM glutamate. After five minutes, the media was replaced with fresh sMEM and cultures were incubated with compound 2 or vehicle as appropriate. Cell death was assessed by the LDH assay described in point 10. Results, as shown in Exhibit 2, indicate that compound 2 increases culture survival. Bars represent the mean ± SEM for n=30 wells/conditions. *$p<0.001$ vs. control (injured, untreated) using an unpaired t-test.

12. To show the protective effects of the claimed compounds on neurons, an *in vitro* model of ischemia (stroke)-oxygen/glucose deprivation was conducted using compound 2. Oxygen-glucose deprivation assays are routinely used in the art to test the protective effect of compounds against neuronal injury. *See, e.g.*, Vink *et al.*, 1991, Amer. J. Physiol. 261:R1527-R1532.

Ischemic Injury (Oxygen-glucose Deprivation). Cultures, prepared as described in point 10, were transferred to an anaerobic chamber and washed twice (14 exchanges) with deoxygenated balanced salt solution (BSS) (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1.0 mM $NaHPO_4$, 26.2 mM $NaHCO_3$, 0.01 mM Glycine, 1.0 mg/L phenol red). Cultures were treated with either compound 2 or vehicle and incubated under anaerobic conditions for seventy minutes. Thereafter, cultures were washed again with fresh, deoxygenated BSS and removed from the chamber. At this point, oxygen-glucose deprivation was halted by the addition of an equal volume of oxygenated BSS supplemented

with 5.5 mM glucose and compound 2 or vehicle as appropriate. Cell death was assessed by the LDH assay described in point 10. Exhibit 3 shows that compound 2 exhibits a protective effect, as evidenced by decreased LDH release.

13. The protective effects of the disclosed compounds were additionally tested using a staurosporine model of apototic cell death. Staurosporine induces apototic cell death in neuronal culture systems. *See, e.g.*, Koh *et al.*, 1995, Exp. Neurology 135:153-159. The assays were performed using compounds 1 and 2. Cells prepared as described in point 10 were washed once (sMEM) and incubated with 0.3 μM staurosporine in the presence and absence of the designated concentrations of compound 1 or 2. Results, as illustrated in Exhibits 4 and 5, demonstrate that treatment with either compound 1 or 2 increases survival in the model. Bars represent mean ± SEM for n=30 wells/conditions. Additional data not shown indicates that compound 2 reduces the number of cells with condensed or fragmented nuclei visualized with Hoechst 33258. *$p<0.0001$ vs. control (injured, untreated) using unpaired t-test.

14. The protective effects of the disclosed compounds were additionally tested in a traumatic injury model. The traumatic injury model is an art accepted model for testing the neuroprotective effects of compounds. *See, e.g.*, Mukhin *et al.*, 1998, J. Neurosci. Res. 51:748-758. Cultures, prepared as described in point 10, were washed (sMEM) once and incubated with or without compound for 30 minutes. Cells were then injured with a mechanical punch that delivered 38 parallel, uniformly distributed cuts to the surface of the cell layer. Thirty minutes after injury, cultures were washed again (sMEM) and incubated with compound 2 or vehicle as appropriate. Results, as illustrated in Exhibit 6, indicate that

compound 2 increases survival of neuronal cells after traumatic injury consisting of a series of parallel cuts to the culture surface. In Exhibit 6, data represent mean ± SEM for n = 30 wells/conditions. *p<0.05 using unpaired t-test.

15. The protective effects of the disclosed compounds were additionally tested in a necrotic injury model. Cultures, prepared as described in point 10, were washed (sMEM) once and incubated with 0.1 nM maitotoxin for one hour in the presence or absence of compound 2. Maitotoxin is a polyether marine algal toxin that causes a rapid and profound increase in intracellular calcium that produces necrotic cell death. Zhao *et al.*, 1999, Neurochem. Res. 24:371-382. At the end of the incubation period, the media is replaced with fresh sMEM with or without compound. Results, as illustrated in Exhibit 7, indicate that compound 2 improved survival in cultures subject to necrotic insult via maitotoxin. Bars indicate the mean ± SEM for n = 30 wells/conditions. *p<0.0001 vs. control (injured, untreated) using unpaired t-test.

16. I declare that all statements made in this Declaration are of my own knowledge and are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 10/15/99

ALAN I. FADEN, M.D.